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EXPERIMENTAL ARTICLES

Microbial Biodiversity in the Water of Lake Baikal

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Abstract—An investigation of the microbial community of Lake Baikal by the methods of general and molecular microbiology showed that culturable bacterial strains are represented by various known genera. The lake water contains a great number of bacterial morphotypes, as revealed by electron microscopy, and a great diversity of nonculturable microorganisms belonging to different phylogenetic groups, as revealed by 16S rRNA gene fragment sequencing. The inference is made that the microbial community of Lake Baikal contains not only known species but also new bacterial species that are possibly endemic to the lake.

Key words: Lake Baikal, total bacterial number, biodiversity, 16S rRNA.

There is increasing interest in the study of aquatic microbial communities, since, being very small organisms, microbes comprise an essential part of the biomass in various bodies of water. The high growth rate and good adaptability of microorganisms allow them to dwell in different habitats. In the early 1990s, the novel method of 16S rRNA gene sequencing made it possible to demonstrate the existence of new bacteria, whose nucleotide sequences formed specific clusters on phylogenetic trees [1]. Later, another approach to the study of bacterial communities in situ with the aid of oligonucleotide probes was proposed [2, 3]. A comparative analysis of microorganisms cultivated on selective media and those detected by sequencing showed that different groups of microorganisms can be detected by these approaches [4].

The study of the microbial community of Lake Baikal by different methods [5–9] provided a great deal of information on the number and the distribution of total microorganisms, heterotrophic bacteria, and other particular bacterial groups in the water and sediments of Lake Baikal [5, 6]. In the 1990s, molecular biological studies of nonculturable bacteria were started. A water sample taken from a depth of 1200 m exhibited a great diversity of novel nucleotide sequences [7]. The suggestion that different depths of the lake are dominated by different bacterial groups was confirmed by the results of the experiments in which water samples were taken at the central station in the southern basin of Lake Baikal from different depths, from the water surface to the bottom layer [9]. These experiments showed that cyanobacteria and actinobacteria were dominant in the surface water and at a depth of 400 m, respectively, whereas the water at a depth of 1200 m contained representatives of almost all groups of proteobacteria. The deep water also contained cyanobacteria, but their nucleotide sequences were different from those detected in the surface water.

This study was aimed at investigating the biodiversity of microorganisms in the water of Lake Baikal by the methods of general and molecular microbiology.

MATERIALS AND METHODS

Water sampling and fixation. The lake water was sampled in the 1996 summer season at the central stations of three transects: Listvyanka settlement–Tankhoi settlement (the southern lake basin); Cape Ukhan–Cape Tonkii (the middle lake basin); and Baikal'skoe village–Cape Turali (the northern lake basin). To enumerate the total microorganisms, water samples were fixed with glutaraldehyde added to a final concentration of 1%. To obtain the total bacterial DNA samples, 1–2 l of the lake water was passed through a 0.22-μm Millipore filter. The residue was washed off from the filter and suspended in a 10 mM Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl. The suspension was centrifuged, and the resultant pellet was frozen and stored at –20°C.

Electron microscopic studies of bacterial morphotypes. Water samples (2 l) were kept in a refrigerator for 1 day. An aliquot (50–100 ml) of bottom water, which was enriched in bacterial cells due to their precipitation, was concentrated by filtration. Bacterial suspension (approximately 1 ml) was transferred on a grid, evaporated for 6–8 h, and fixed with formalin vapor. Whole cell specimens were contrasted with 2% phosphotungstic acid (pH 7.0) and examined in a JEM-100C transmission electron microscope (Japan).

Total bacterial count by epifluorescence microscopy. The microorganisms present in the water samples were stained with the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI), which binds to DNA molecules. A water sample was mixed with a dye solution to give

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Table 1. The total bacterial abundance and the number of culturable heterotrophic bacteria in the water of Lake Baikal investigated during the 1996 summer season

Transect	Depth, m	TBA, million cells/ml	Heterotrophs, cells/ml
Listvyanka–Tankhoi (the southern basin)	0	4.6	310
	5	3.9	315
	10	3.8	290
	25	1.8	280
	35	2.1	170
	50	1.0	62
	100	0.8	60
	200	0.6	35
	500	0.2	17
Ukhan–Tonkii (the middle basin)	0	3.9	200
	5	4.5	220
	10	4.7	130
	25	2.2	110
	50	1.3	90
	150	1.1	66
	250	0.8	40
	500	0.4	34
	1000	0.15	10
	1500	0.17	12
Baikal'skoe–Turali (the northern basin)	0	3.2	575
	5	3.2	310
	10	2.4	140
	25	2.1	55
	50	1.7	30
	100	0.8	12
	250	0.6	7
	500	0.4	15
	800	0.2	26

a final concentration of the dye equal to 0.5 μg/ml. The mixture was kept for 1 min and passed through a 0.2-μm Nuclepore filter (PC). The filter was washed with sterile water, placed onto a specimen slide, and examined in an Olympus epifluorescence microscope with an oil-immersion objective.

The cultivation of heterotrophic bacteria. Heterotrophic bacteria were isolated using fish meal–peptone (1 : 10) agar plates (FPA plates) inoculated with water samples in a proportion of 1–5 ml water per 25 ml medium. After 3–7 days of incubation at room temperature, the colonies grown on the plates were enumerated, and various bacterial morphotypes were isolated. The taxonomic position of isolates was determined from their morphological, physiological, and biochemical properties, using the identification criteria of Bergey's Manual [10].

Table 2. The species composition of culturable heterotrophic bacteria dominated at different depths in the water of the Lake Baikal southern basin during the 1996 summer season

Depth, m	Number of colonies	Species
0	49	*Planococcus citreus*
25	100	*Aeromonas achromogenes*
400	32	*Acinetobacter calcoaceticus*
1000	24	*Pseudomonas synxantha*
1200	35	*Pseudomonas fluorescens*
1400	30	*Bacillus alvei*

DNA isolation. Bacterial DNA was isolated using a QIAamp Blood&Tissue kit (QIAGENE, United States) according to the manufacturer's instructions.

Primers. The primers used in the work were complementary either to the most conservative regions of 16S rRNA genes (parenthesized are the *E. coli*–based positions of nucleotides) or (in the case of plasmid primers) to the conservative regions of plasmid pAT123 [9]:

500L, CGTGCCAGCAGCCGCGGTAA (514–533);

800L, AGGATTAGATACCCTGGTAGTC (790–812);

1000L, GATGCAACGCGAAGAACCTTACC (972–994);

1000R, CCTGGTAAGGTTCTTCGCGTTGC (975–997);

1230R, CATTGTAGCTCGTGTGTAGCCC (1219–1240);

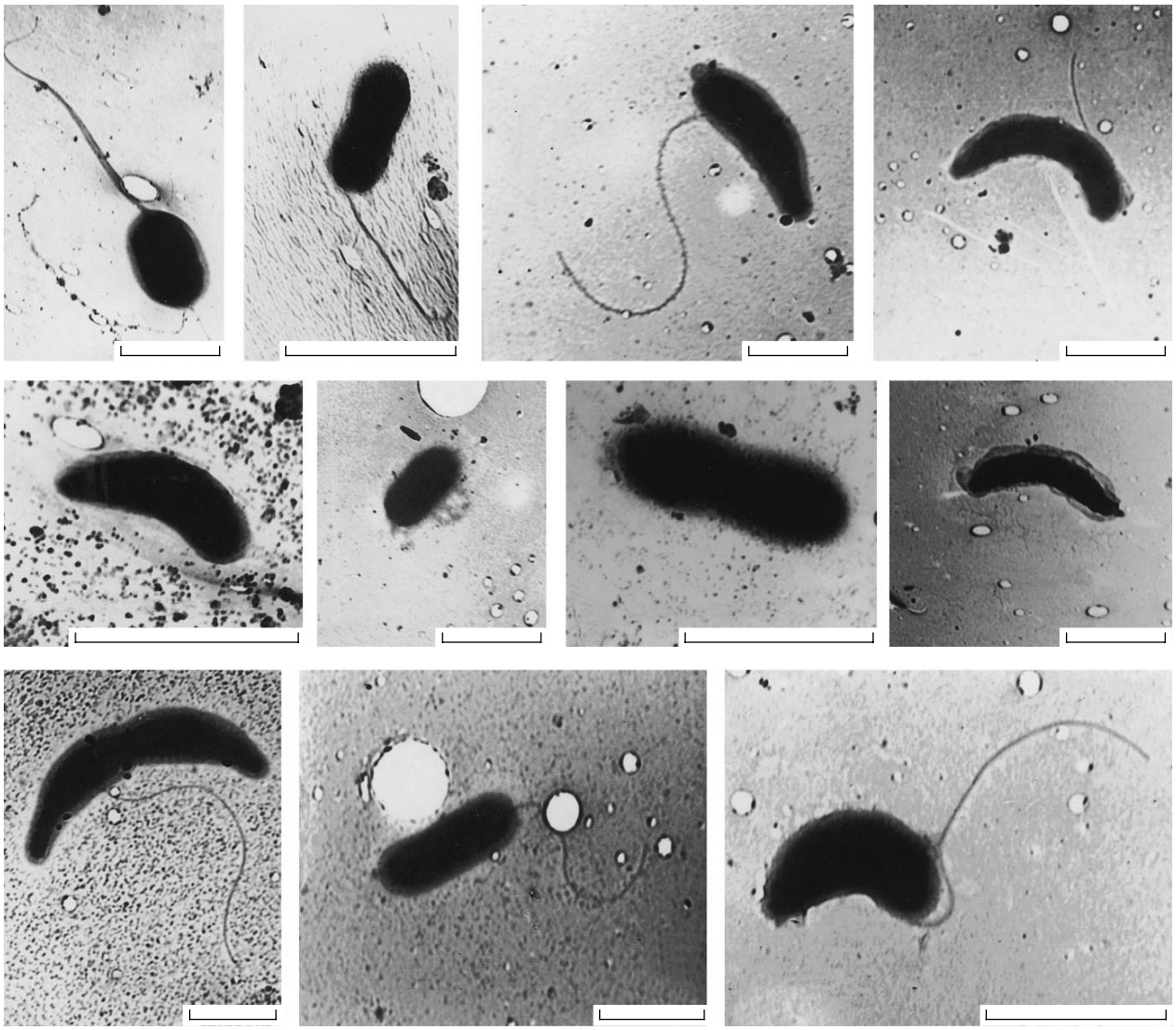
1350R, GACGGGCGGTGTGTACAAG (1389–1407);

M13-UP, GGAAACAGCTATGACCAT (plasmid pAT123);

M13-DOWN, GTAAAACGACGGCCAGTG (plasmid pAT123).

The amplification of 16S rDNA fragments. The total bacterial DNA was used as a template in the polymerase chain reaction with the oligonucleotide primers 500L and 1350 R, which was performed under conditions that provided for the greatest diversity of nucleotide sequences [9].

Cloning the PCR products and analysis of the clones. The PCR products containing 16S rRNA gene fragments were cloned using the ClonTech plasmid pAT123, which is able to efficiently insert DNA fragments. Ligation and transformation procedures were carried out by the standard methods [11]. Clones were



(a)

Fig. 1. Bacterial morphotypes detected in the water samples taken in the (a) middle and (b) northern basins of Lake Baikal. The scale bars represent 1 μm.

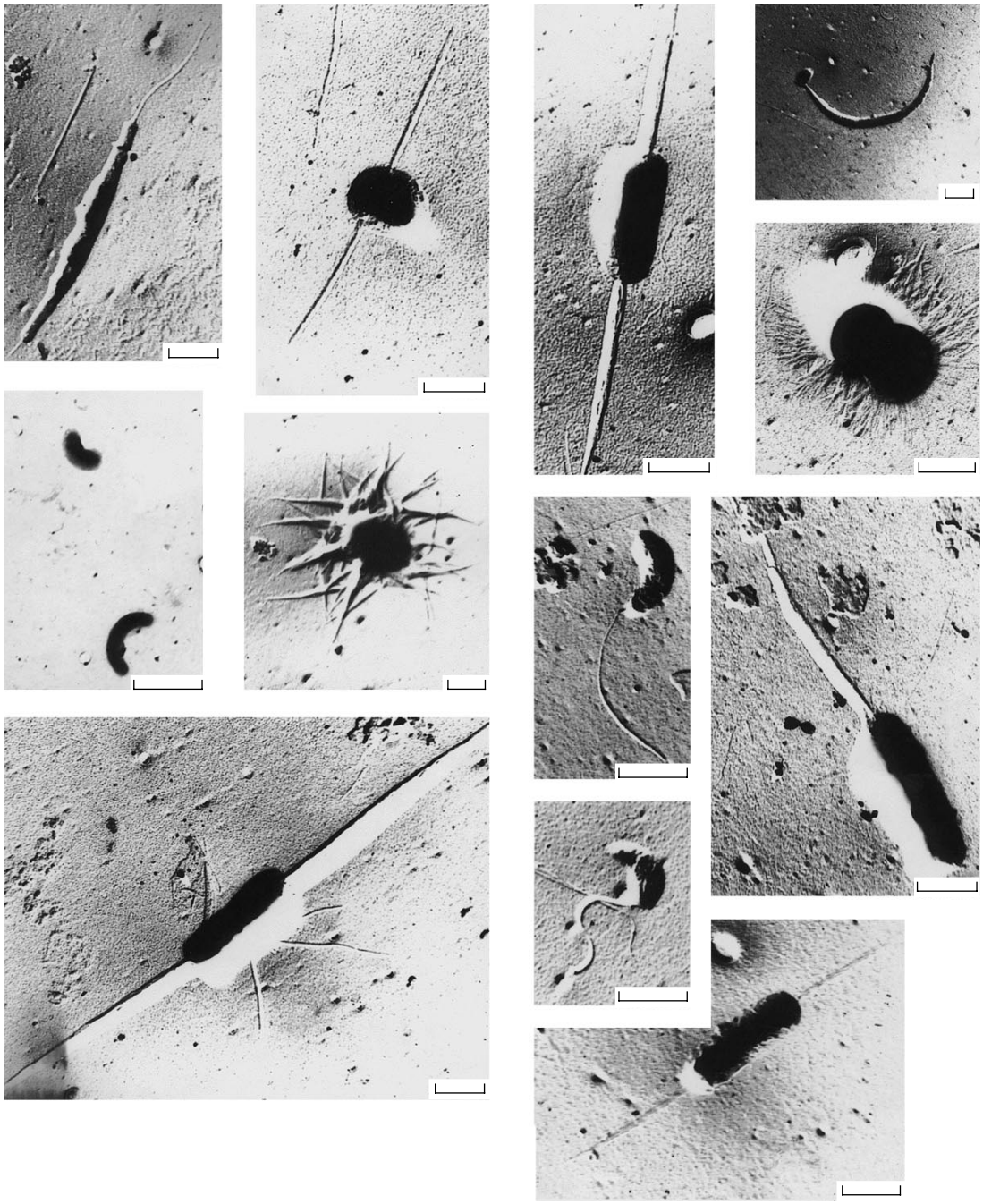
selected by the white–blue screening procedure and analyzed by PCR using a suspension of heat-inactivated cells (2 μl) as a DNA template and primers complementary to the plasmid pAT123 regions in the vicinity of the insertion (M13-UP and M13-DOWN).

16S rRNA gene fragment sequencing. The PCR products obtained with the primers complementary to the 16S rRNA gene positions 500 and 1000 were purified by electrophoresis in agarose gel and sequenced using a PCR Cyclist kit purchased from Stratagene (United States). Sequencing products were separated by electrophoresis in 7% polyacrylamide gel containing 8 M urea and visualized by radiography. Further sequencing steps were carried out and identical clones were elucidated by the approach described earlier [9].

Analysis of nucleotide sequences. The sequences derived were compared with those in the EMBL database with the aid of the FASTA program. Phylogenetic trees were generated using TREECON W software [12].

RESULTS AND DISCUSSION

The total bacterial population determined by epifluorescence microscopy included cells with a size of 0.2 μm or larger. The results are presented in Table 1. The total bacterial abundance (TBA) in the water samples taken from the southern basin of Lake Baikal ranged from 0.2×10^6 to 4.6×10^6 cells/ml. In the same water samples, the number of heterotrophs grown on FPA plates varied from 17 to 315 cells/ml. In the water of the middle basin, the TBA varied from 0.15×10^6 to 3.9×10^6 cells/ml, and the number of culturable heterotrophs varied from 10 to 220 cells/ml. Correspondingly, the TBA in the water of the northern basin varied from 0.2×10^6 to 3.2×10^6 cells/ml, and the number of



(b)

Fig. 1. (Contd.)

culturable heterotrophs varied from 7 to 575 cells/ml. The decrease in the TBA with depth correlated with the decrease in the number of heterotrophs. The maximum amounts of total and heterotrophic bacteria were detected in the surface layer and at depths of 5–10 m. Considerable differences in the numbers of total and culturable bacteria were also observed by other researchers for other natural ecosystems [2, 4]. It is beyond doubt that the biodiversity of an aquatic microbial community cannot be entirely characterized by only the taxonomic range of culturable heterotrophic bacteria, although this is an essential characteristic of the whole microbial community.

The culturable heterotrophic bacteria of Lake Baikal were found to belong to the genera *Bacillus, Pseudomonas, Arthrobacter, Micrococcus, Acinetobacter, Alcaligenes, Escherichia, Flavobacterium, Xanthomonas, Corynebacterium*, and *Vibrio*. The heterotrophic bacterial species that dominated at different depths in the water of southern Baikal are presented in Table 2. These data confirm the high diversity of microorganisms in Lake Baikal [13] and in other bodies of water.

Table 3. Bacterial clones in the water of middle Baikal taken from a depth of 25 m and bacteria in the EMBL database that are the closest in the 16S rRNA gene sequences

Clone	Occurrence rate	Closest bacterium in EMBL (% similarity)	Phylogenetic group
25-5-2	1	Unidentified cyanobacterial clone LD9 (98.1%)	Cyanobacteria
25-5-3L	7	Unidentified cyanobacterial clone LD9 (99.6%)	Cyanobacteria
25-5-11	1	Unidentified cyanobacterial clone LD9 (98.4%)	Cyanobacteria
25-5-20	1	Unidentified proteobacterium arc 53 (99.3%)	β-Proteobacteria
25-5-35	2	Unidentified cyanobacterial clone LD9 (96.7%)	Cyanobacteria
25-5-61	1	Unidentified cyanobacterial clone LD9 (98.3%)	Cyanobacteria
25-5-64	1	Unidentified actinomycete ACK-M1 (94.1%)	Actinobacteria
25-5-66	1	Unidentified actinomycete ACK-M1 (93.9%)	Actinobacteria
25-5-66L	1	*Sporichthya polymorpha* (90.7%)	Actinobacteria
25-5-69	1	Unidentified actinomycete ACK-M1 (91.3%)	Actinobacteria

Table 4. Bacterial clones in the water of middle Baikal taken from a depth of 1400 m and bacteria in the EMBL database that are the closest in the 16S rRNA gene sequences

Clone	Occurrence rate	Closest bacterium in EMBL (% similarity)	Phylogenetic group
1405-1L	1	*Methylobacter psychrophilus* (94.7%)	γ-Proteobacteria
1405-2	4	Lucina pectinata (symbiont) (92.3%)	γ-Proteobacteria
1405-9L	1	Unidentified β-proteobacterium OPB37 (92.6%)	β-Proteobacteria
1405-10	1	Soil bacterial clone SC-I–55 (89.7%)	δ-Proteobacteria
1405-15	1	Eubacterial clone M7 from Amazonia (87.5%)	Proteobacteria
1405-19L	1	Nonculturable bacterium from manganese nodules (98.3%)	*Nitrospira* group
1405-20	1	*Acinetobacter johnsonii* (99.1%)	γ-Proteobacteria
1405-22	1	Nonculturable soil bacterial clone C112 (97.2%)	*Holophaga* group
1405-25	1	Nonculturable *Methylobacter* pAMC419 (91.3%)	γ-Proteobacteria
1405-41	1	Nonculturable bacterium GKS2-103 (94.6%)	Actinobacteria
1405-48	1	Nonculturable *Methylobacter* pAMC419 (95.1%)	γ-Proteobacteria
1405-49L	1	Bacterial clone kb2426 (96.7%)	*Holophaga* group
1405-50	1	Nonculturable bacterium GKS2-103 (88.9%)	Actinobacteria
1405-51	1	Nonculturable bacterium GKS2-103 (91.3%)	Actinobacteria
1405-55	1	*Streptomyces griseus* (86.1%)	Actinobacteria
1405-56	1	*Sphingomonas* sp., clone BF14 (98.6%)	α-Proteobacteria
1405-57	1	Nonculturable bacterium ARFS-33 (92.8%)	Actinobacteria
1405-59	1	*Sphingomonas* sp., clone BF14 (95.0%)	α-Proteobacteria
1405-60	1	*Sphingomonas* sp., clone BF14 (95.5%)	α-Proteobacteria
1405-61	1	Nonculturable bacterium ARFS-33 (89.3%)	Actinobacteria
1405-62	1	Soil bacterial clone SC-I-86 (97.2%)	*Holophaga* group
1405-69	2	*Sphingomonas* sp., clone BF14 (93.3%)	α-Proteobacteria
1405-72L	1	Nonculturable bacterium FukuN30 (97.5%)	Actinobacteria
1405-79	1	*Rhizosphere* soil bacterial clone RSC-II-60 (96.7%)	β-Proteobacteria
1405-86	1	Nonculturable bacterium GKS2-103 (92.7%)	Actinobacteria

Table 5. Bacterial clones in the water of middle Baikal taken from a depth of 1650 m and bacteria in the EMBL database that are the closest in the 16S rRNA gene sequences

Clone	Occurrence rate	Closest bacterium in EMBL (% similarity)	Phylogenetic group
1605-3	3	α-Proteobacterial clone 33 (95.8%)	α-Proteobacteria
1605-6	3	*Aeromicrobium erythreum* (87.3%)	Actinobacteria
1605-10	1	Unidentified actinomycete clone ACK-M1 (93.4%)	Actinobacteria
1605-13L	2	Unidentified eubacterial clone DA008 (89.2%)	*Acidiphilum* group (low gc)
1605-13	2	Unidentified eubacterium RB07 (92.1%)	*Acidiphilum* group (low gc)
1605-17	3	*Pandoraea pulmonicola* (94.0%)	β-Proteobacteria
1605-21	1	Nonculturable β-proteobacterium SBRA220 (87.2%)	β-Proteobacteria
1605-22	1	*Thiobacillus aquaesulis* (92.4%)	β-Proteobacteria
1605-23	1	Nonculturable bacterial clone 404-8 (99.3%) *Rhodoplanes elegans* (96.2%)	α-Proteobacteria
1605-23L	1	*Synechococcus* PCC9005 (87.7%)	α-Proteobacteria
1605-26	2	*Streptomyces griseus* (86.4%)	Actinobacteria
1605-28	1	Unidentified eubacterium RB07 (91.8%)	*Acidiphilum* group (low gc)
1605-35	1	Nonculturable bacterium BURTON-14 (88.1%)	Actinobacteria
1605-43	1	Nonculturable bacterial clone *Methylobacter* pAMC419 (95.6%)	γ-Proteobacteria
1605-50	2	*Riftia pachyptila* symbiont (90.5%)	γ-Proteobacteria
1605-54	1	Unidentified actinomycete ACK-M1 (89.9%)	Actinobacteria
1605-56	1	Unidentified actinomycete ACK-M1 (91.8%)	Actinobacteria
1605-59L	1	*Methylobacter psychrophilus* (95.5%)	γ-Proteobacteria

Light and epifluorescence microscopy, commonly used to study the morphological diversity of bacteria, allow only rods, cocci, vibrios, and, sometimes, spiral and yeastlike cells to be distinguished. At the same time, electron microscopy makes it possible to reveal a strikingly high diversity of bacterial morphotypes isolated from different depths. The micrographs presented in Figs. 1a and 1b exemplify the bacterial morphotypes revealed in the lake water by the direct microscopic examination of water samples. Noteworthy is the fact that these samples contain a great number of motile cells with flagella, fimbriae, pili, and other threadlike appendages, which is in agreement with the observations of Lapteva [13]. Surprisingly, these atypical morphotypes are not detected when bacteria are cultivated on the standard nutrient media.

The 16S rRNA gene fragment sequence analysis of three water samples taken at the central station of middle Baikal from depths of 25, 1400, and 1600 m revealed the presence of 74 different clones, which were divided into 53 groups based on the results of the so-called G-ladder sequencing. One of the clones in each of these groups was completely sequenced, and these sequences were compared with those available in the EMBL database. The results of these comparisons are presented in Tables 3–5. The phylogenetic relations between our clones and the closest homologues found in the EMBL database are shown in Figs. 2–4. The nonculturable bacteria of middle Baikal are represented by cyanobacteria, proteobacteria, and gram-positive actinobacteria, as well as by several representatives of the *Holophaga* and *Nitrospira* groups (Fig. 2) [14]. As can be seen from Tables 3–5, most of the sequences derived were homologous to those of aquatic nonculturable microorganisms. For instance, of the ten sequences derived from the analysis of the lake water sample taken from the 25-m depth (Table 3), only one sequence was homologous to that of the soil actinomycete *Sporichthya polymorpha*, whereas the nine other sequences were highly homologous to those of aquatic cyanobacteria (five sequences), actinobacteria (three sequences), and proteobacteria (one sequence). All of the reference sequences were obtained for nonculturable microorganisms of freshwater ecosystems [15, 16]. Many nucleotide sequences obtained from the analysis of the lake water samples taken from depths of 1400 and 1650 m were highly homologous to those of nonculturable bacteria (Tables 4, 5). Of the 25 sequences typical of the depth 1400 m, 21 sequences were highly homologous to nonculturable representatives of different proteobacterial and actinobacterial subgroups and *Nitrospira* and *Holophaga* groups. Of the 18 sequences typical of the depth 1650 m, only 7 were highly homologous to the known bacterial species. Within each of the groups, the degree of sequence homology was within the range typical of species differences. Five of the ten sequences presented in Table 3 were highly homologous (from 96.7 to 99.6%) to that of the cyano-

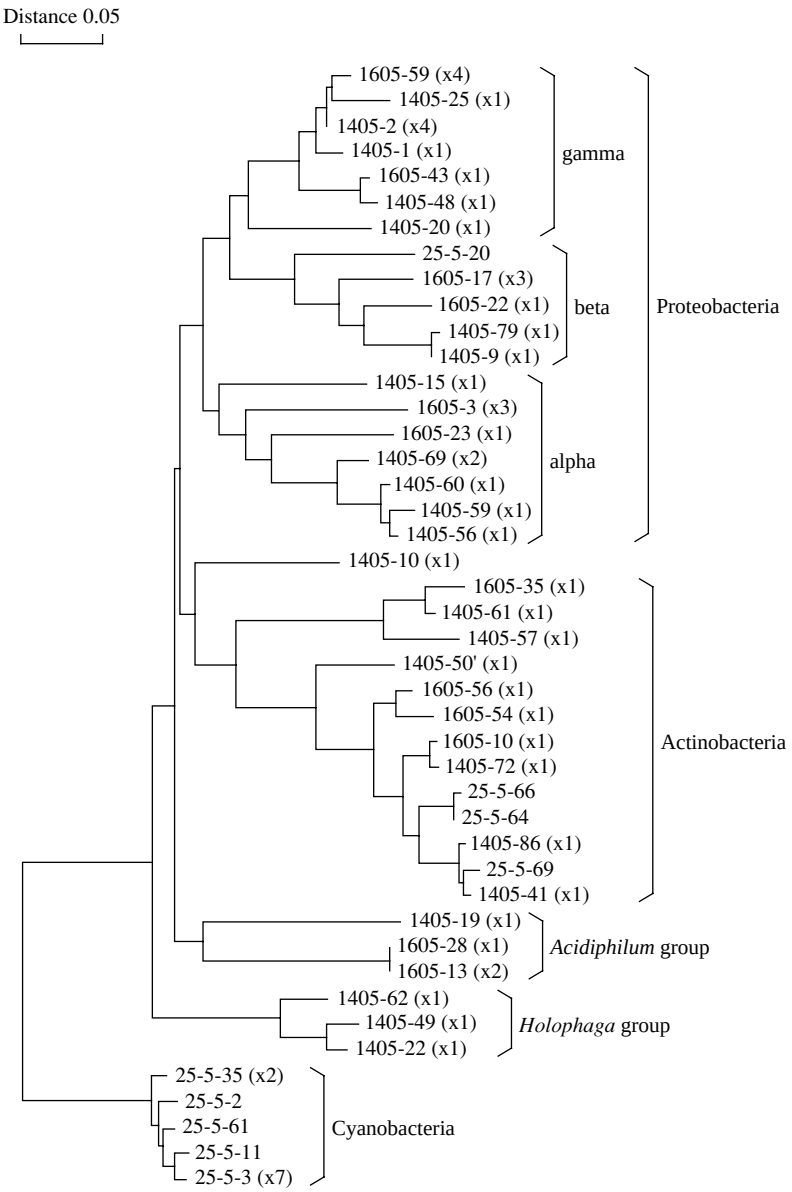


Fig. 2. A phylogenetic tree generated from the 16S rRNA gene sequences of bacteria detected in the water of Lake Baikal. Parenthesized are the numbers of clones having a particular nucleotide sequence.

bacterial clone LD9. However, most of the sequences typical of the depth 1400 m (Table 4) had a low homology to those in the database: four showed 93.3–98.6% homology to that of the nonculturable *Sphingomonas* sp. clone BF14, and the four others showed 88.9–94.6% homology to the nonculturable actinobacterium GKS2-103. Five sequences (three typical of the depth 25 m and two typical of the depth 1650 m) showed 90–93% homology to that of the closest microorganism, unidentified actinomycete ACK-M1, which is presently ascribed to a new phylogenetic cluster, hgcI, in the family *Actinomycetaceae* [17].

On the phylogenetic trees presented in Figs. 3 and 4, the nucleotide sequences of microorganisms occurring in the water of middle Baikal are clustered together with those of microorganisms from other aquatic ecosystems [18–20]. Low-depth waters (25 m) in Lake Baikal are dominated by cyanobacteria, whose nucleotide sequences form phylogenetic clusters with those obtained earlier for southern Baikal [9] and with cyanobacteria from the subgroup *Synechococcus.* Deep-water bacteria are mainly represented by proteobacteria and gram-positive bacteria. The proteobacteria of middle Baikal are clustered with the proteobacteria of southern Baikal and some other nonculturable aquatic and soil proteobacteria (Fig. 3). In the gamma subgroup, the nucleotide sequences typical of the water of Lake Baikal are clustered with the nucleotide sequences obtained for the bottom samples of other

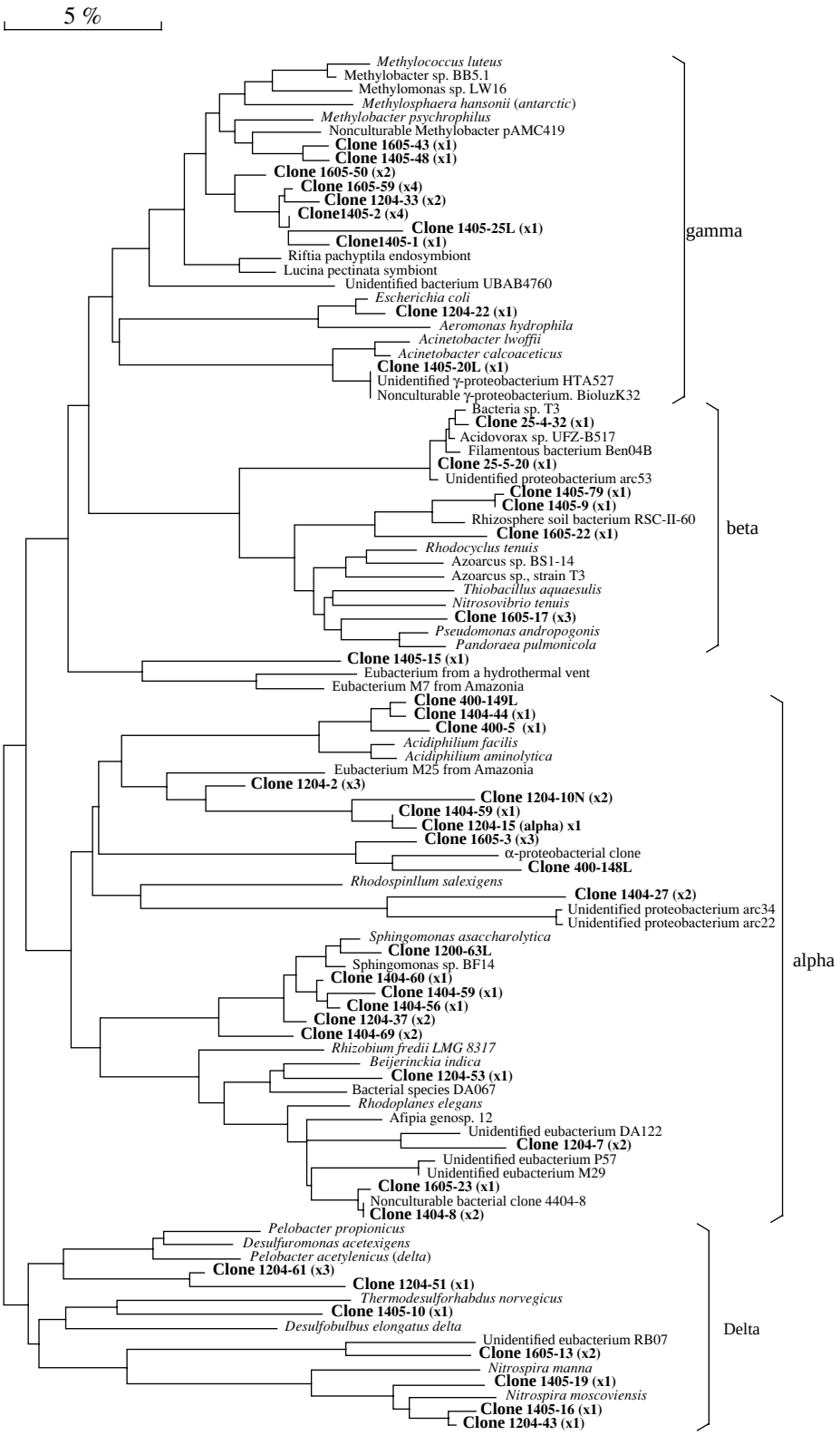


Fig. 3. A phylogenetic tree generated for proteobacteria detected in the water of Lake Baikal and those in the EMBL database that are the closest in the 16S rRNA gene sequences. Clones revealed in the water of middle Baikal in this work are given in bold type, while clones revealed earlier in the water of southern Baikal [9] are given in italic type.

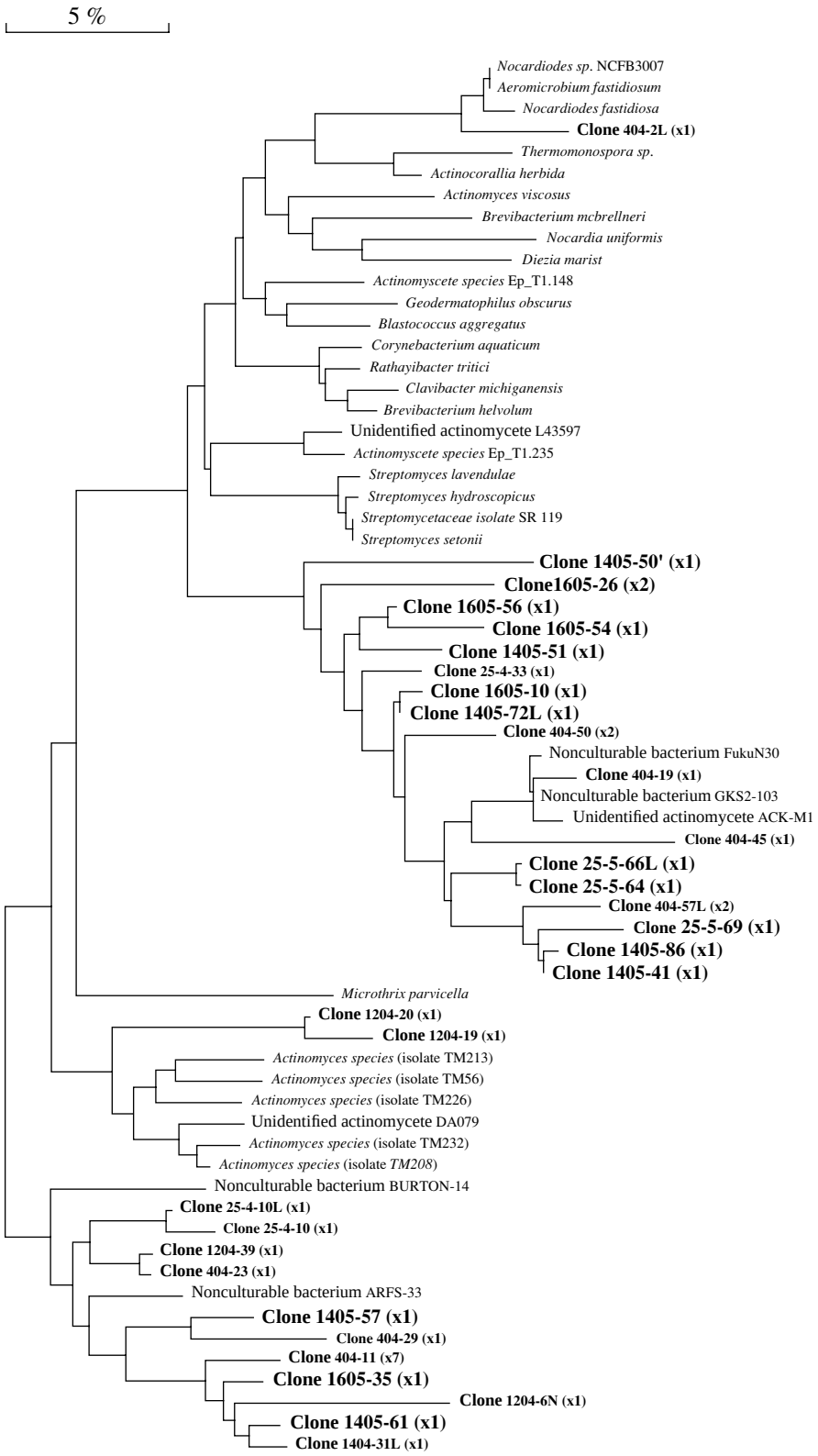


Fig. 4. A phylogenetic tree generated for actinobacteria detected in the water of Lake Baikal and those in the EMBL database that are the closest in the 16S rRNA gene sequences.

bodies of water [19, 20]. Distinct endogenous clusters were also observed in the alpha subgroup (the *Acidiphilum*, *Sphingomonas*, and Clone 33 clusters) and in the *Nitrospira* group associated with delta-proteobacteria [14]. The gram-positive actinomycetes of Lake Baikal form two clusters (Fig. 4), one of which contains the nonculturable bacteria ARFS33 and BURTON-14 and is close to a cluster formed by nonculturable soil and aquatic bacteria. The other cluster includes the nonculturable bacteria GKS2-103 and FukuN30 from lakes Gossenkoellesee (Austria) and Fuchskuhle (Germany), which form the aforementioned cluster hgcI of natural aquatic bacteria in the phylogenetic group of actinobacteria.

Thus, this study considerably broadens our knowledge of the diversity of culturable and nonculturable microorganisms occurring in the water of Lake Baikal.

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